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RESEARCH ARTICLE

DETECTION OF *BLA_{KPC-2}* GENE AMONG CARBAPENEM-RESISTANT *SERRATIA MARCESCENS* ISOLATES FROM EGYPT.

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Abstract

Background: *Serratia marcescens* is a common healthcare-associated pathogen affecting several body sites. The emergence of carbapenem-resistant *S. marcescens* strains in the healthcare settings is a matter of great clinical concern.

Objective: This study was conducted to detect the frequency of *Bla_{KPC-2}* gene encoding for *Klebsiella pneumoniae* carbapenemase-2 (KPC-2) among carbapenem-resistant *S. marcescens* isolates.

Materials and Methods: A prospective cohort study was conducted over a period of 12 months starting from December 2014 to November 2015. Clinical specimens were collected from patients admitted to different medical centers in Mansoura University. *S. marcescens* isolates were identified based on colony morphology, Gram staining characters, and standard biochemical reactions. Antimicrobial susceptibility testing was determined by the disk diffusion method. The modified Hodge test (MHT) was performed for detection of carbapenemase production by *S. marcescens* isolates. Genetic detection of *Bla_{KPC-2}* gene among carbapenem-resistant *S. marcescens* isolates was validated by polymerase chain reaction (PCR).

Results: During the study period, a total of 23 *S. marcescens* isolates were recovered from different clinical specimens. 17 isolates (74%) exhibited either resistance or reduced susceptibility to the tested carbapenems. The MHT was positive in 76.5% of the carbapenem non-susceptible *S. marcescens* isolates. PCR amplicons revealed the presence of *Bla_{KPC-2}* gene in 12 out of the 17 (70.6%) carbapenem resistant *S. marcescens* isolates.

Conclusion: Carbapenem-resistant *S. marcescens* due to KPC-2 represents a leading threat in healthcare facilities. So, effective measures should be endorsed to prevent the potential propagation of these pathogens.

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Introduction:-

Serratia marcescens is a ubiquitous, motile, non-endospore forming Gram-negative bacillus that belongs to the family *Enterobacteriaceae* (Yu, 1979). Cardinal features of *S. marcescens* include; the production of DNase, lipase and gelatinase, and it is oxidase negative. This bacterium grows well on standard bacteriologic media and produces a red to dark pink pigment called prodigiosin that assists in its identification (Hejazi and Falkiner, 1997).

Although *S. marcescens* is considered an opportunistic pathogen, it has emerged as an important healthcare-associated organism. This bug is implicated in wide range of deliberate infections including; urinary, respiratory, and biliary tract infections, peritonitis, wound infections, and intravenous catheter-related infections, which can lead to life-threatening bacteraemia (Mahlen, 2011).

S. marcescens has high resistance to β -lactams, aminoglycosides, and fluoroquinolones. There are different mechanisms for resistance of *S. marcescens* to β -lactam drugs including; the production of inactivating enzymes (β -lactamases), the constitutive expression of efflux pumps, low permeability of its outer membrane, and alteration of penicillin-binding proteins (PBPs) targets. In *S. marcescens*, all probable mechanisms predisposing to resistance to β -lactams may exist concurrently or in various combinations in clinical isolates (Yang et al., 2012).

Carbapenems are crucial for the treatment of life-threatening infections and often considered the antimicrobials of last resort to treat infections caused by extended-spectrum β -lactamase (ESBL)-producing organisms (Papp-Wallace et al., 2011). However, distinct class A and B carbapenemases are contributed to carbapenem resistance in *S. marcescens*. Many chromosomally encoded SME-type class A carbapenemases have been detected in *S. marcescens* strains (Queenan et al., 2000). Furthermore, the plasmid-borne class B metallo- β -lactamases (MBLs), including IMP-type variants (Osano et al., 1994) and VIM-2 (Yum et al., 2002) have been described in *S. marcescens*.

In addition, the plasmid-mediated carbapenemase, *Klebsiella pneumoniae* carbapenemase-2 (KPC-2), has been recognized in carbapenem-resistant *S. marcescens* isolates (da Costa Guimarães et al., 2013). The carbapenem-hydrolyzing KPC β -lactamases belong to molecular class A. They are capable of hydrolyzing carbapenems, penicillins, cephalosporins and aztreonam, and are inhibited by clavulanic acid and tazobactam (Chen et al., 2014).

The purpose of this study was to investigate the frequency of *Bla*_{KPC-2} gene among carbapenem-resistant *S. marcescens* isolates recovered from different medical centers in Mansoura University.

Materials and Methods:-

This prospective cohort study was conducted over a period of 12 months starting from December 2014 to November 2015. The study design was approved by the Local Ethics Committee.

Specimens Collection:-

Clinical specimens were collected from the patients in various medical centers in Mansoura University including; the Main Mansoura University Hospital, Gastroenterology Center (GEC), Specialized Internal Medicine Hospital, Oncology Center of Mansoura University (OCMU), and Emergency Hospital.

Processing of Specimens:-

The processing of different specimens was performed in the microbiology laboratory at the Microbiology Diagnostics and Infection Control Unit (MDICU), Faculty of Medicine, Mansoura University. All media used in the study were purchased from Oxoid (Basingstoke, UK) and prepared according to the manufacturer's instructions. *S. marcescens* isolates were identified based on colony morphology, Gram staining characters, and biochemical reactions (Koneman et al., 1997).

Antimicrobial Susceptibility Testing:-

Antimicrobial susceptibility was determined by Kirby-Bauer's disk diffusion method according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2014). Bacterial inocula were prepared by suspending the freshly grown bacteria in normal sterile saline then adjusted to a 0.5 McFarland standard. Antimicrobial disks (Oxoid, Basingstoke, UK) included; piperacillin/tazobactam (100/10 μ g), ceftazidime (30 μ g), ceftriaxone (30 μ g), cefotaxime (30 μ g), cefepime (30 μ g), cefoperazone/sulbactam (75/30 μ g), aztreonam (30 μ g), imipenem (10 μ g), meropenem (10 μ g), amikacin (30 μ g), gentamicin (10 μ g), ciprofloxacin (10 μ g), levofloxacin (5 μ g), and trimethoprim/sulfamethoxazole (1.25/23.75 μ g). *E. coli* ATCC 25922 was used for quality control.

Screening of ESBLs-Producing *S. marcescens* Strains:-

This was done as a part of the routine susceptibility testing, according to the criteria recommended by the CLSI (CLSI, 2014). Two disks, ceftazidime (30 µg) and cefotaxime (30 µg), were used. Strains showing zone of inhibition of ≤ 22 mm for ceftazidime, and ≤ 27 mm for cefotaxime were selected for conformational tests of ESBL.

Phenotypic Confirmatory Test for ESBLs Production:-

Production of ESBLs was detected by the double-disk synergy test (DDST) (Jarlier et al., 1988), with the disk of amoxicillin–clavulanic acid (20/10 µg) [Oxoid, Basingstoke, UK] placed at the center of Muller–Hinton agar (MHA) plates. At both sides of amoxicillin–clavulanic acid disk, disks of cefotaxime (30 µg) and ceftazidime (30 µg) were placed with center to center distance of 30 mm to the centrally placed disk. The plates were incubated at 37°C overnight. ESBLs production was interpreted if cefotaxime and/or ceftazidime disk inhibition zone increased towards the amoxicillin–clavulanic acid disk.

Determination of the Minimum Inhibitory Concentration (MIC) of Carbapenems:-

The MICs of the tested carbapenems (imipenem and meropenem) were determined using the agar dilution method in accordance with the CLSI guidelines (CLSI, 2014). Strains with imipenem and/or meropenem MICs ≥ 2 µg/ml were further characterized using phenotypic tests.

Screening for Carbapenemase Production by the Modified Hodge Test (MHT):-

The modified Hodge test (MHT) was used for detection of carbapenemases according to the CLSI recommendations using *E. coli* ATCC 25922 (CLSI, 2011). Briefly, the surface of MHA plate was inoculated evenly with a suspension of *E. coli* ATCC 25922 (1:10 dilution of turbidity adjusted to 0.5 McFarland). After brief drying, a disk containing 10 µg imipenem was placed at the center of the plate and carbapenem-resistant isolates from overnight culture plates were streaked from the edge of the disk to the plate periphery. The test was considered positive if a distorted inhibition zone (a characteristic cloverleaf-like indentation) was observed around the imipenem disk after overnight incubation at 37°C.

Genetic Detection of *Bla*_{KPC-2} among Carbapenem-Resistant *S. marcescens* Isolates by PCR:-

Genomic DNA was extracted from *S. marcescens* isolates from an overnight broth cultures using the method described by Chen and Kuo (Chen and Kuo, 1993). PCR amplifications of *Bla*_{KPC-2} were performed using TaqPCR Master Mix (AM, Egypt), and the *Bla*_{KPC-2} primers (AM, Egypt); 5'-TGT CAC TGT ATC GCC GTC-3' (F), 5'-CTC AGT GCT CTA CAG AAA ACC-3' (R). PCR reaction mixtures (25 µl volume) contained; 1 µl DNA template, 12.5µl TaqPCR Master Mix, 1 µl of each primer and 9.5 µl of nuclease-free water. The reactions were amplified in a thermal cycler (MJ Research, Inc., USA). Cycling parameters were 5 min at 95°C, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 30 s, and extension at 72°C for 1 min 30 s. The PCR amplification was ended by a final extension cycle at 72°C for 10 min (Yigit et al., 2001). The PCR products were visualized by electrophoresis in 1.5% agarose gels stained with ethidium bromide under ultraviolet transilluminator (Fisher Scientific, Pittsburg, CA, USA). Gels were photographed (photo-documentation) using digital camera.

Conjugation Experiment:-

The conjugation experiment was done according to the method described by Wang et al. (Wang et al., 2003). The mating process was performed in Luria-Bertani (LB) broth using sodium azide-resistant *E. coli* J53 as the recipient. The recipient and donor strains were cultured in 4 ml fresh LB broth and incubated overnight without shaking at 37°C. Transconjugants were selected on trypticase soy agar plates supplemented with sodium azide (100 µg/ml; Sigma Chemical Co., St. Louis, MO) and meropenem (0.5µg/ml). Colonies growing on trypticase soy agar plates supplemented with meropenem were subjected to the MHT to confirm the presence of carbapenemase-producing transconjugants. Moreover, PCR was done for *E. coli* transconjugants to verify the acquisition of *Bla*_{KPC-2} gene.

Statistical Analysis:-

The statistical analysis was performed using SPSS (Statistical Package for the Social Sciences) version 20.0 for Windows. Qualitative data were described in the form of number and percentage. Medians were used to describe asymmetrically distributed continuous variables. A $p < 0.05$ was considered to be statistically significant.

Results:-

During the study period from December 2014 to November 2015, a total of 205 clinical isolates were recovered from various kinds of clinical specimens including; sputum, endotracheal aspirate (ETA), bronchoalveolar lavage (BAL), throat swabs, ascetic fluid, cerebrospinal fluid (CSF), urine, blood, wound swabs, and pus. Twenty three (23) *S. marcescens* strains were isolated from different medical centers of Mansoura University (Figure 1) representing 11.2% of the total isolates. Identification of *S. marcescens* was based on their colony characters, Gram staining reaction, and biochemical reactions being positive for DNase, lipase, gelatin liquifaction, and lysine and ornithine decarboxylases, while negative for indole and oxidase tests.

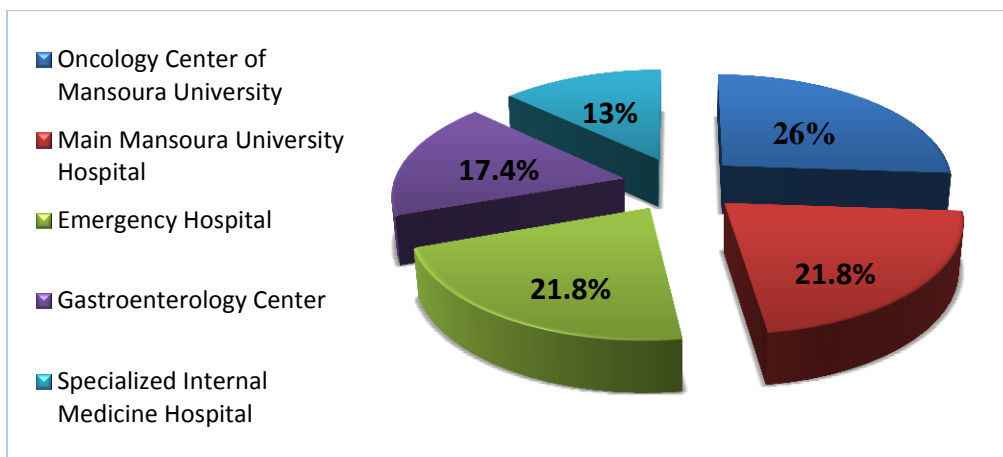


Figure 1: Distribution of *S. marcescens* Isolates from Different Medical Centers in Mansoura University.

Antimicrobial Susceptibility Testing:-

In vitro susceptibility testing revealed that (Table 1), the highest sensitivity of the tested *S. marcescens* isolates was to piperacillin-tazobactam, levofloxacin, and amikacin (78.3%, 73.9%, and 69.6%, respectively). On the other hand, 47.8% and 43.5% of the isolates were sensitive to meropenem and imipenem, respectively. There was no statistically significant difference in the percentages of *S. marcescens* isolates susceptible to meropenem and imipenem (47.8% versus 43.5%; $p = 0.4$). Furthermore, only 26.1% and 21.7% of the isolates showed sensitivity to ceftazidime and cefotaxime, respectively.

Table 1: Antibiotic Susceptibility Profile of *S. marcescens* Isolates by Disk Diffusion Test.

Antibiotic	Susceptible strains	
	Number (23)	Percent (100%)
Piperacillin-tazobactam	18	78.3
Levofloxacin	17	73.9
Amikacin	16	69.6
Cefoperazone-sulbactam	13	56.5
Ciprofloxacin	13	56.5
Meropenem	11	47.8
Imipenem	10	43.5
Gentamicin	10	43.5
Aztreonam	10	43.5
Cefepime	10	43.5
Ceftriaxone	9	39.1
Trimethoprim/sulfamethoxazole	8	34.8
Ceftazidime	6	26.1
Cefotaxime	5	21.7

Phenotypic Confirmatory Test for ESBLs Production by DDST:-

Out of the 23 tested *S. marcescens* isolates, 78.3% of the isolates (n=18) were found to be phenotypically ESBLs-producers according to the criteria set by the CLSI (Jarlier et al., 1988).

Determination of the MIC of Carbapenems:-

Of the 23 investigated *S. marcescens*, 17 isolates (74%) exhibited either resistance or reduced susceptibility to the tested carbapenems with imipenem and/or meropenem MICs of 2 to 64 µg/ml (median: 8 µg/ml).

Screening for Carbapenemase Production by the MHT:-

The MHT (Figure 2) results for imipenem and/or meropenem resistant strains (n=17) demonstrated that 76.5% of the carbapenems non-susceptible isolates (n=13) had the carbapenemase phenotype.

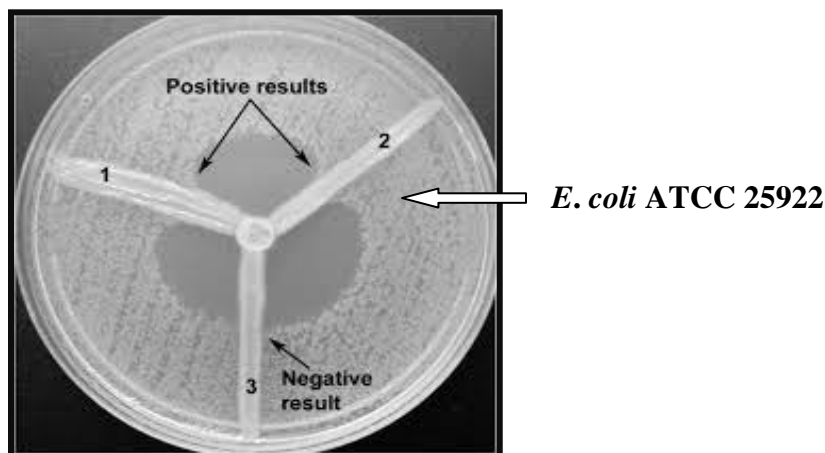


Figure 2: The Modified Hodge Test (MHT) on Muller Hinton Agar Plate.

1. Control strain: Positive result "a characteristic cloverleaf-like indentation around the imipenem disk".
2. *S. marcescens* strain: Positive result.
3. *S. marcescens* strain: Negative result.

Genetic Detection of *Bla*_{KPC-2} among Carbapenem-Resistant *S. marcescens* Isolates by PCR:-

PCR was carried out for the identification of KPC-producing isolates by amplification of *Bla*_{KPC-2} gene in the 17 carbapenems non-susceptible strains. The PCR products revealed that 12 out of the 17 carbapenems non-susceptible *S. marcescens* isolates (70.6%) harbored the *Bla*_{KPC-2} gene (Figure 3). *S. marcescens* strains carrying the *Bla*_{KPC-2} gene were distributed in all tested Mansoura University medical centers where 41.7% and 25% of them were recovered from the oncology center and the Main hospital, respectively (Table 2) more frequently from sputum samples (Table 3).

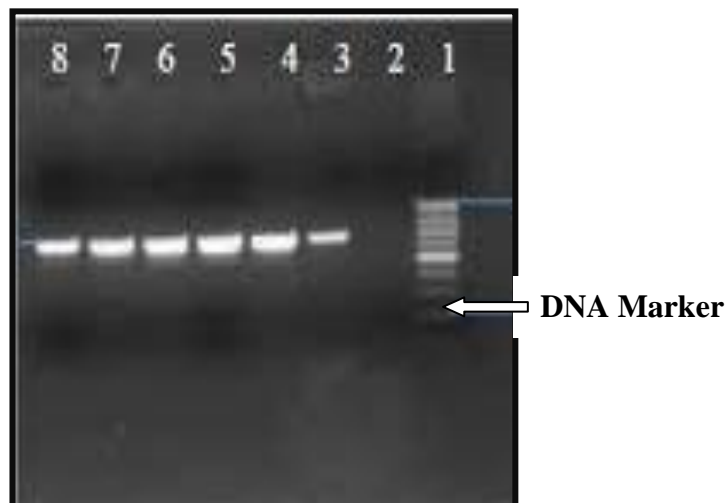


Figure 3: PCR Analysis of *Bla*_{KPC-2} Gene from Carbapenem Resistant *S. marcescens* Isolates.

The amplicons (~900bp) were separated by agarose gel electrophoresis. **Lane 1:** DNA standard marker, Φ X 174 - HaeIII digest, marker with fragments ranging in size from 1353 bp to 72 bp. **Lane 2:** Negative result for *Bla*_{KPC-2} gene. **Lanes 3 to 8:** *S. marcescens* isolates have *Bla*_{KPC-2} gene.

Table 2: Distribution of *S. marcescens* Isolates with *Bla*_{KPC-2} from Different Medical Centers in Mansoura University.

Medical Centers	Total <i>S. marcescens</i> Isolates (n=23)	<i>Bla</i> _{KPC-2} Positive <i>S. marcescens</i> Isolates (n=12)
OCMU	6 (26.0%)	5 (41.7%)
MMUH	5 (21.8%)	3 (25%)
EH	5 (21.8%)	2 (16.7%)
GEC	4 (17.4%)	1 (8.3%)
SIMH	3 (13.0%)	1 (8.3%)

OCMU: Oncology Center of Mansoura University; MMUH: Main Mansoura University Hospital; EH: Emergency Hospital; GEC: Gastroenterology Center; SIMH: Specialized Internal Medicine Hospital.

Table 3: *S. marcescens* Strains Isolated from Different Clinical Specimens.

Specimen type	Total <i>S. marcescens</i> Isolates (n=23)	<i>Bla</i> _{KPC-2} Positive <i>S. marcescens</i> Isolates (n=12)
Sputum	7 (30.4%)	3 (25%)
Wound Swabs	5 (21.7%)	2 (16.7%)
Blood	3 (13.0%)	2 (16.7%)
Pus	3 (13.0%)	2 (16.7%)
BAL	2 (8.7%)	1 (8.3%)
Urine	2 (8.7%)	1 (8.3%)
ETA	1 (4.3%)	1 (8.3%)

BAL: Bronchoalveolar lavage; ETA: Endotracheal aspirate.

Conjugal Transfer of Carbapenems Resistance:-

The recipient strain *E. coli* J53 was subjected to conjugation with the 12 *Bla*_{KPC-2} gene positive *S. marcescens* isolates as donor strains. All of the *Bla*_{KPC-2} positive *S. marcescens* isolates (100%) had been conjugated successfully as indicated phenotypically by the growth of *E. coli* transconjugants on trypticase soy agar plates supplemented with sodium azide and meropenem. All the *E. coli* transconjugants (n=12) had reduced carbapenems susceptibility with imipenem and/or meropenem MICs of 2 to 32 µg/ml (median: 6 µg/ml). In addition, all *E. coli* transconjugants yielded positive results for *Bla*_{KPC-2} gene.

Discussion:-

The first description of nosocomial infections caused by *S. marcescens* was reported by Wheat et al. in 1951 (Wheat et al., 1951). Since 1960s, infections caused by this bacterium have been recorded with increasing density (Dodson, 1968). Such infections constitute a tangible cost as regard to patients' morbidity and mortality.

Carbapenem-resistant *S. marcescens* has been frequently reported worldwide (Cai et al., 2008; Silva et al., 2015). It represents a hospital challenge as it causes problems regarding the therapy and control. Therefore, investigation of the molecular mechanisms of carbapenem resistance is crucial. The aim of this study was to verify the frequency of *Bla*_{KPC-2} gene encoding for carbapenem non-susceptibility among *S. marcescens* isolates recovered from various clinical specimens obtained from different medical centers in Mansoura University.

During the study period from December 2014 to November 2015, 23 non-duplicate *S. marcescens* isolates were retrieved from 23 different patients (only one isolate/patient) causing 11.2% of nosocomial infections comparable to another Egyptian study where *S. marcescens* accounted for 7.14% of infections in the studied patients (Shehab El-Din et al., 2015).

In the current study, *S. marcescens* isolates were recovered from 13 males (56.5%) and 10 females (43.5%) with age ranging between 19 and 67 years (median; 30 years). The patients were selected from different medical centers of Mansoura University with length of hospital stay ranging from 7 to 58 days (median; 17 days).

By disk diffusion test, 78.3%, 73.9%, and 69.6% of the isolates (n=23) were sensitive to piperacillin-tazobactam, levofloxacin, and amikacin, respectively. ESBLs- *S. marcescens* producers were detected in 78.3% of the isolates. On the other hand, 52.2% and 56.5% of the isolates were resistant to meropenem and imipenem,

respectively with no statistically difference between them. In contrast to these findings, Silva et al. demonstrated that 100% of *S. marcescens* strains isolated from Brazilian teaching hospital were resistant to imipenem and meropenem (Silva et al., 2015) while, Liou and co-workers observed that 99.3% of their *S. marcescens* isolates were sensitive to imipenem (Liou et al., 2014). This discrepancy could be attributed to different epidemiological features of the enrolled patients, different used antibiotic regimens in the studied hospitals as well as the use of multiple antimicrobial susceptibility testing techniques.

The most widely used phenotypic test for detection of carbapenemases, and the only recommended one by the CLSI is the MHT (Cury et al., 2012). In the present work, 76.5% of the carbapenem non-susceptible isolates had the carbapenemase phenotype. The negative MHT results obtained in 23.5% of carbapenem-resistant *S. marcescens* isolates propose the involvement of other resistance mechanisms. Carbapenem resistance may develop as a result of production of carbapenemases (KPC, NDM, OXA, and M β L) alone or in combination with the loss of porins (Doumith et al., 2009), ESBL (TEM, SHV, CTX-M) and/or AmpC enzymes associated with porin loss, and the presence of efflux pumps (Fehlberg et al., 2012).

In the present study, we evaluated *S. marcescens* isolates with reduced susceptibility or resistance to carbapenems for the presence of genes encoding for carbapenem resistance. Out of the 17 carbapenem-resistant *S. marcescens* isolates, 12 (70.6%) were demonstrated to harbor the *Bla*_{KPC-2} gene by the PCR. In a different study, a higher frequency of this gene (90.5%) was detected by Zhou and associates (19 out of 21 *S. marcescens* isolates harbored the *Bla*_{KPC-2} gene) (Zhou et al., 2013). This difference could be due to the use of other molecular technique in their study (pulsed-field gel electrophoresis (PFGE)) to verify the existence of this gene. In addition, Zhang et al. demonstrated the presence of *Bla*_{KPC-2} gene in 3 clinical carbapenem-resistant *S. marcescens* isolates from a neonatal intensive care unit (NICU) in a Chinese hospital in February 2006 (Zhang et al., 2007). Soon afterward, 21 *Bla*_{KPC-2} positive *S. marcescens* isolates were recovered from the same ward from April 2006 to February 2007 (Cai et al., 2008).

In our study, it is possible that other genes encoding for carbapenem resistance, not detected by the PCR, were present in the other 29.4% carbapenem-resistant *S. marcescens* isolates. Additionally, it is well known that, despite the standardization of the MHT for detection of carbapenemases, it can't differentiate between their different types and can give false positive results with other enzymes, CTX-M types ESBLs, so it is recommended to use the MHT or a boronic acid screening test for carbapenemases phenotyping in combination with PCR for detection of *Bla*_{KPC-2} gene (Eftekhari and Naseh, 2015).

In the present work, notably, 25% of the *Bla*_{KPC-2} positive *S. marcescens* strains were isolated from sputum samples, 16.7% from surgical wound swabs, 16.7% from blood cultures, 8.3% from BAL, 8.3% from urine specimens, and 8.3% from ETA.

Our data revealed that, following mating of the 12 *Bla*_{KPC-2} positive *S. marcescens* isolates with *E. coli* J53, all the resulting transconjugants yielded positive results for *Bla*_{KPC-2} gene by the PCR denoting successful transfer of this gene by conjugation. Similar results were achieved in different literatures (Zhang et al., 2007; Cai et al., 2008; Silva et al., 2015). This finding is worrisome because it reflects the possibility of transmission of *Bla*_{KPC-2} gene of carbapenem resistance encoded on a conjugative plasmid, particularly in clinical settings, among different bacterial genera leaving clinicians with a paucity of effective antimicrobial agents.

Conclusion:-

This study reveals the presence of *Bla*_{KPC-2} gene in 70.6% of carbapenem-resistant *S. marcescens* isolates which contributes to the understanding of the molecular mechanisms underlying carbapenem resistance in this organism. Therefore, special attention must be taken steadily regarding the emergence and spread of these pathogenic microbes, as well as the implementation of effective strategies for the prevention and control of dissemination of these pathogens.

Source of Support, Conflict of Interest: none.

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